PPARs as new therapeutic targets for the treatment of cerebral ischemia/reperfusion injury

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Abstract

Stroke is a leading cause of death and long-term disability in the industrialized countries. Despite advances in understanding its pathophysiology, little progress has been made in the treatment of stroke. The currently available therapies have proven to be highly unsatisfactory (except thrombolysis) and attempts are being made to identify and characterize signalling proteins which could be exploited to design novel therapeutic modalities. The peroxisome proliferators activated receptors (PPARs) are ligand-activated transcription factors that control lipid and glucose metabolism. PPARs regulate gene expression by binding with the retinoid X receptor (RXR). RXR as a heterodimeric partner to specific DNA sequences, termed PPAR response elements. In addition, PPARs may modulate gene transcription also by directly interfering with other transcription factor pathways in a DNA-binding independent manner. To date, three different PPAR isoforms, designated α, β/δ and γ, have been identified. Recently, they have been found to play an important role for the pathogenesis of various disorders of the central nervous system and accumulating data suggest that PPARs may serve as potential targets for treating ischemic stroke. Activation of all PPAR isoforms, but especially of PPARγ, was shown to prevent post-ischemic inflammation and neuronal damage in several *in vitro* and *in vivo* models, negatively regulating the expression of genes induced by ischemia/reperfusion (I/R). This paper reviews the evidence and recent developments relating to the potential therapeutic effects of PPAR-agonists in the treatment of cerebral I/R injury.

Cerebral ischemia/reperfusion injury

Stroke has a major impact on the public health of every nation. Ischemic stroke, which accounts for more than 80% of all stroke events, results from a transient or permanent reduction in cerebral blood flow that, in most cases, is caused by thrombotic occlusions. Ischemic damage of the central nervous system (CNS) may also present the clinical picture of a transient ischemic attack (TIA), which affords for a less severe neurological deficit in comparison with stroke. Ischemic stroke is the second leading cause of death in Europe and a common cause of long-term disability worldwide. (Frizzel, 2005). Cerebral ischemia is defined as a reduction in cerebral blood flow (CBF), sufficient to cause metabolic or functional deficit. The characteristics of brain injury depends on the severity and the duration of CBF reduction. Although reperfusion following transient ischemia leads to restoration of CBF, there is compelling evidence to support the notion that reperfusion may exacerbate the injury initially caused by ischemia, producing a so-called "cerebral ischemia/reperfusion (I/R) injury". In some animal stroke models, reperfusion after a long ischemic period has been demonstrated to cause a larger infarct than that associated with permanent vessel occlusion (Aronowski et al., 1997; Yang et al., 1994). Thrombolisis, which leads to restoration of cerebral perfusion and at present is the only therapeutic strategy clinically used in most parts of the world, is not without risk (NINDS, 1995). For instance, the thrombolytic agent tissue-type plasminogen activator (tPA) can increase the risk of symptomatic brain hemorrhage (NINDS, 1997), has a brief 3 h time window of efficacy, and is capable of directly causing damage to neurons (Nicole, 2001; Wang, 1998). Thus, while reperfusion may improve clinical outcomes in some patients, in others it may substantially contribute to the pathogenesis of the disease.

The major pathogenic mechanisms of cerebral I/R injury include glutamate-mediated excitotoxicity, oxidative stress, inflammation, necrotic and apoptotic cell death and gene expression (Mehta et al., 2007). These events occur in an overlapping manner and depend on the intensity and duration of the insult. Drugs that can interfere with one or more of these mechanisms might minimize the subsequent neurodegeneration, thus leading the emergence of new therapeutic interventions in cerebral I/R. However, in spite of the growing understanding of the mechanisms of neuronal damage and death accompanying brain I/R, effective therapy has remained elusive (Green and Shuaib, 2006). Recent discoveries portray Peroxisome Proliferator-Activated Receptors (PPARs) as promising pharmacological targets for the treatment of acute ischemic stroke.

PPARs as nuclear receptors

PPARs are members of the nuclear hormone receptor (NHR) superfamily of ligand-activated transcription factors. There are three PPAR subtypes: α, β/δ and γ, named also NR1C1, NR1C2 and NR1C3, respectively, according to the unified nomenclature of nuclear receptors (Nuclear Receptors Nomenclature Committee, 1999). The three isoforms are the products of distinct genes: the human PPAR-α gene was mapped on chromosome 22 in the general region 22q12–q13.1, the PPAR-γ gene is located on chromosome 3 at position 3p25, whereas PPAR-β/δ has been assigned to chromosome 6, at position 6p21.1–p21.2 (Greene et al., 1995; Sher et al., 1993; Yoshikawa et al., 1996). PPARs were originally identified by Isseman and Green (1990) after screening the rat liver cDNA library with a cDNA sequence located in the highly conserved C domain of NHRs. These investigators demonstrated that chemicals that act as peroxisome proliferators were potent ligands for this new nuclear receptor, hence its designation as PPAR-α. Activation of neither PPAR-β/δ nor PPAR-γ, however, elicits this response and, interestingly, the phenomenon of peroxisome proliferation does not occur in humans (Vamecq and Draye, 1989). The molecular basis for this difference between species is not yet clear. With respect to the PPAR-γ isotype, alternative splicing and promoter use results in the formation of two further isoforms: PPAR-γ1 and PPAR-γ2. In particular, differential promoter usage and alternate splicing of the gene generates three mRNA isoforms. PPAR-γ1 and PPAR-γ3 mRNA both encode the PPAR-γ1 protein product which is expressed in most tissues, whereas PPAR-γ2 mRNA encodes the PPAR-γ2 protein, which contains an additional 28 amino acids at the amino terminus and is specific to adipocytes (Gurnell, 2003). All members of this superfamily share the typical domain organization of nuclear receptors. The N-terminal A/B domain contains a ligand-independent transactivation function. In the α and γ isotypes, the activity of this domain can be regulated by mitogen-activated protein kinase (MAPK) phosphorylation (Juge-Aubry et al., 1999; Hu et al., 1996). The C domain is the DNA binding domain with its typical two zinc-finger-like motifs, as previously described for the steroid receptors (Schwabe et al., 1990). The E/F domain is the ligand binding domain. It contains a ligand-dependent trans-activation function (AF)-2 (Fajas et al., 1997), and is able to interact with transcriptional coactivators such as steroid receptor coactivator (SRC)- 1 (Kalkhoven et al. 1998; Krey et al., 1997; Onate et al., 1995) and CREB-binding protein (CBP) (Chakravarti et al., 1996; Dowell et al., 1997; Kamei et al., 1996).

Endogenous and synthetic PPARs ligands

A broad spectrum of natural and synthetic compounds can function as PPAR ligands by binding to PPARs. Although many fatty acids are capable of activating all three PPAR isoforms, some preference for specific fatty acids by each PPAR has been demonstrated. The long-chain polyunsaturated fatty acids and their oxidized derivatives, especially eicosanoids such as 8-S-hydroxyeicosatetraenoic acid (8-S-HETE), leukotriene B_4 (LTB_4) and arachidonate monooxygenase metabolite epoxyeicosatrienoic acids have been shown to potently activate PPAR-α with high affinity (Willson et al., 2000; Feige et al., 2006; Theocharis et al., 2004). PPAR-β/δ agonists include linoleic acid, oleic acid, arachidonic acid and eicosapentaenoic acid (EPA), which have been shown to co-crystallize within the ligand binding domain of this nuclear receptor (Xu et al., 1999). A number of eicosanoids, including prostaglandin (PG) A_1 and PGD_2, and carbaprostacyclin, a semi-synthetic prostaglandin, have micromolar affinities for PPAR-β/δ (Forman et al., 1997). PPAR-γ can be activated by several prostanoids, such as 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) and 12- and 15-hydroxy-eicosatetraenoic acid (12- and 15-HETE), which are derivatives of arachidonic acid synthesized through the lipoxygenase pathway, as well as modified oxidised lipids, 9- and 13-hydroxyoctadecadienoic acids (9- and 13-HODE) (Willson et al., 2000; Theocharis et al., 2003; Theocharis et al., 2004). The cyclopentenone PG 15d-PGJ2 is not only the most potent natural ligand for PPAR-γ identified to date, but also by far the most commonly used naturally occurring PPAR-γ agonist (Forman et al., 1995). It was first discovered in 1983, following incubation of PGD2 for extended periods of time in the presence of albumin (Fitzpatrick et al., 1983). However, it received relatively little attention until 1995 when two independent groups simultaneously reported that it is capable of activating PPAR-γ (Forman et al., 1995; Kliewer et al., 1995). Although it is clear that 15d-PGJ2 can stimulate PPAR-γ, the concentrations of 15d-PGJ2 required to stimulate PPAR-γ are generally reported to be in the micromolar range (Powell, 2003). In addition, using a highly sensitive liquid chromatography/tandem mass spectrometry assay for 15d-PGJ2, Bell-Parikh and colleagues reported that although 15d-PGJ2 can be generated in vivo, the levels produced are not sufficient to be compatible with a role for this substance as an endogenous ligand for PPAR-γ (Bell-Parikh et al., 2003). Thus, whether 15d-PGJ2 is the endogenous ligand for PPAR-γ is still not clear. Besides, it is important to note that 15d-PGJ2 can induce a variety of PPAR-γ independent responses, and 15d-PGJ2 has indeed been shown to induce responses in cells devoid of the receptor (Chawla et al., 2001).

With respect to the synthetic ligands, fibrates (e.g. fenofibrate, clofibrate), which are hypolipidaemic drugs, are well-known ligands for PPAR-α (Willson, 2000; Theocharis, 2003; Theocharis, 2004). Fibrates are capable are capable of activating PPAR-α at pharmacological doses

leading to increased expression of lipid metabolizing enzymes that effectively lower serum lipid levels in humans. In contrast to the well-documented therapeutic effect, there is also evidence of liver toxicity induced by activation of PPAR-α, mainly hepatocarcinogenesis. In response to ligand activation by fibrates, PPAR-α mediates increased transcription of acyl-CoA oxidase and other target genes that lead to increased cell proliferation in the liver (reviewed in Klaunig et al., 2003; Peters et al., 2005). Fibrates can also interfere with aryl hydrocarbon receptor (AhR)-dependent signaling. Expression of the cytochrome P450 (CYP) 1A2 and enzyme activity in liver are both decreased in rats treated with ciprofibrate (Gallagher et al., 1995). Similarly, decreased expression of CYP1A1 and CYP1A2 mRNA and protein is found in rat liver after clofibrate treatment, and this effect appears to be due to reduced turnover of the AhR that mediates induction of CYP1A1 and CYP1A2 (Shaban et al., 2004). These combined observations suggest that PPAR-α ligands could potentially inhibit bioactivation and/or detoxification of chemical carcinogens/toxicants catalyzed by CYPs and, at the same time, increase cell proliferation, thus leading to hepatocellular carcinomas. However, clinical trials have failed to show an increase in cancer diagnoses between treatment groups (Keech et al., 2005; Rubins et al., 1999). The most serious safety risk associated with fibrates, although rare, is myopathy and rhabdomyolysis (Gaist et al., 2001). Studies suggest that the mechanism of myotoxicity through fibrates is not entirely clear, because complex and multifactorial mechanisms are involved, including genetic predisposition, pharmacokinetics, drug interactions, and dose. It is of interest to note that increased expression of lipoprotein lipase, which is known PPAR-α target gene, in skeletal muscle leads to severe myopathy in mice (Levak-Frank et al., 1995; Schoonjans et al., 1996).

On the other hand, synthetic ligands for PPAR-β/δ are currently in preclinical phases of study: their safety and their therapeutic potential for obesity, dyslipidemia and type-2-diabetes is now under investigation in *in vivo* experimental models (Takahashi et al., 2006).

The most widely used PPAR-γ agonists belong to the thiazolidinedione (TZD) or glitazone class of anti-diabetic drugs used in the treatment of type-2 diabetes. Troglitazone, the first TZD approved for this use, was withdrawn from the market in March 2000 following the emergence of a serious hepatotoxicity in some patients. Since troglitazone induces CYP3A4 (Dimaraki and Jaffe, 2003; Ramachandran et al., 1999), it has been hypothesized that potentially toxic quinones derived from CYP3A4-dependent metabolism could cause liver damage (Neuschwander-Tetri et al., 1998; Yamamoto et al., 2002). The two currently available TZDs, rosiglitazone and pioglitazone, were approved in the United States in 1999 and are currently used alone or in combination with other oral anti-diabetic agents for type-2 diabetes patients (Willson et al, 2000; Theocharis et al., 2003; Theocharis et al., 2004; Margeli et al, 2003). On the basis of evidence from clinical trials and post-

marketing experience, rosiglitazone and pioglitazone do not appear to be associated with hepatotoxicity. However, there are side effects common to all TZDs, which can be deemed a class effect. One of the most studied toxic effects of TZDs is cardiac toxicity, mainly increased plasma volume leading to edema, which in turn can exacerbate congestive heart failure (Patel et al., 2005). This increase in fluid volume appears to be mediated by PPARγ-dependent expression of renal epithelial sodium channel (Guan et al., 2005b; Zhang et al., 2005). In the last few years, a large number of studies have revealed a broad spectrum of action for the TZD class of drugs beyond the treatment of diabetes, including anti-inflammatory and anti-neoplastic properties, as well as their critical role in atherosclerosis and various CNS diseases (Theocharis et al., 2003; Theocharis et al., 2004; Margeli et al., 2003; Hamerman et al., 2005; Pershadsingh et al., 2004). Currently, a new generation of dual-action PPAR ligands, such as muraglitazar and tesaglitazar, are also being developed to activate both PPAR-α and PPAR-γ in order to combine their anti-diabetic actions with reducing diabetic complications (Pershadsingh et al., 2006).

Transcriptional activities of PPARs

Mechanisms of transcriptional transactivation

PPARs function as heterodimers with their obligate partner — the retinoid X receptor (RXR). Like other NHRs, the PPAR/RXR heterodimer probably recruits co-factor complexes — either co-activators or co-repressors — that modulate its transcriptional activity (Surapureddi et al.,2002; Mueller et al., 2002; Krogsdam et al., 2002; Shi et al., 2002) The PPAR/RXR heterodimer then binds to sequence specific PPAR response elements (PPREs), located in the 5'-flanking region of target genes, thereby acting as a transcriptional regulator (Forman et al., 1995; Palmer et al., 1995; Varanasi et al., 1996; Zhang et al., 1996). In the absence of a ligand, to prevent PPAR/RXR binding to DNA, high-affinity complexes are formed between the inactive PPAR/RXR heterodimers and co-repressor molecules, such as nuclear receptor co-repressor or silencing mediator for retinoic receptors. Upon binding an agonist, the conformation of a PPAR is altered and stabilized such that a binding cleft is created and recruitment of transcriptional co-activators occurs. The result is an increase in gene transcription. The search for PPAR target genes with identified PPREs has led to the identification of several genes involved in lipid metabolism, oxidative stress and the inflammatory response, as widely documented in the literature (Berger and Moller, 2002; Desvergne and Whali, 1000; Tan et al., 2005).

Mechanisms of transcriptional transrepression.

PPARs can also negatively regulate gene expression in a ligand-dependent manner by inhibiting the activities of other transcription factors, such as activated protein-1 (AP-1), nuclear factor-κB (NF-κB), nuclear factor of activated T cells (NFAT) or signal transducer and activator of transcription (STAT) (ligand-dependent transrepression). In contrast to transcriptional activation, which usually involves the binding of PPARs to specific response elements in the promoter or enhancer regions of target genes, transrepression does not involve binding to typical receptor specific response elements (Glass et al., 2007; Pascual et al., 2006). Several lines of evidence suggest that PPARs may exert anti-inflammatory effects by negatively regulating the expression of pro-inflammatory genes. To date, several mechanisms have been suggested to account for this activity, but despite intensive investigation, unifying principles remain to be elucidated.

Firstly, competition for limited amounts of essential, shared transcriptional co-activators may play a role in transrepression. In vitro studies have revealed a ligand type-specific direct interaction of PPARs with several transcriptional co-activators, such as SRC-1, TIF2, AIB-1, CBP, p300, TRAP220, and DRIP205 (Kodera et al., 2000). The activated PPAR/RXR heterodimer reduces the availability of co-activators required for gene induction by other transcriptional factors. Thus, without distinct co-factors, transcriptional factors cannot cause gene expression.

Secondly, PPAR/RXR complexes may cause a functional inhibition by directly binding to transcription factors, preventing them from inducing gene transcription (Chung et al., 2000). Ligand-activated PPAR-α has been shown to interfere with DNA binding of both AP-1 and NF-κB activity in interleukin (IL)-1α stimulated smooth muscle cells as measured by IL-6 induction. PPAR-α inhibits the vascular inflammatory response by direct protein–protein interaction with p65 and c-Jun (Delerive et al., 1999a). Similarly, PPAR-γ inhibits lipopolysaccharide (LPS)-stimulated production of IL-12 in macrophages by direct interaction with p65/p50 (Chung et al., 2000). NFAT precipitation experiments in T cells revealed a direct contact with PPAR-γ, with NFAT sequestration accounting for suppression of T cell proliferation and activation (Yang et al., 2000). Both PPAR-α and PPAR-γ ligands interfere with the AP-1 signalling pathway, which mediates endothelin-1 (ET-1) gene expression in endothelial cells (Delerive et al., 1999b). In addition, PPAR-α ligands, induce the expression of the inhibitory protein inhibitor of kappa B (IκB)α in smooth muscle cells and hepatocytes, which sequesters the NF-κB subunits in the cytoplasm and consequently reduces their DNA binding activity (Delerive et al., 2000; Vanden Berghe et al., 2003). Zingarelli and colleagues (2003) demonstrated that PPAR-γ inhibits activation of AP-1 and reduces degradation of IκBα in the lungs, resulting in reduced activation of NF-κB.

Thirdly, PPAR/RXR heterodimers may also inhibit phosphorylation and activation of several members of the MAPK family. In general very little is known about the molecular mechanisms by which PPARs and their ligands modulate kinase activities. In a study carried out in PPAR-$\gamma^{+/-}$ mice, activation of c-Jun-N-terminal kinase (JNK) and p38 in response to 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis was significantly reduced compared with wild-type littermates (Desreumaux et al., 2001). Jones and colleagues (2003) have shown that unliganded PPAR-α suppresses the phosphorylation of p38 MAPK after T-cell stimulation. Recent studies have suggested another mechanism based on co-repressor-dependent transrepression by PPARs. Evidence has been presented in which PPAR-β/δ controls the inflammatory status of macrophages based on its association with the transcriptional repressor BCL-6 (Lee et al., 2003). In the absence of a ligand, PPAR-β/δ sequesters BCL-6 from inflammatory response genes, leading to increased levels of gene expression. In contrast, in the presence of ligand, PPAR-β/δ releases the repressor, which now distributes to NF-κB-dependent promoters and exerts anti-inflammatory effects by repressing transcription from these genes. Other studies have proposed that PPAR-γ may mediate transrepression of a subset of inflammatory response genes in macrophages by preventing the signal-dependent clearance of co-repressor complexes on inflammatory promoters downstream of LPS signaling (Pascual et al., 2005; Ghisletti et al, 2007). A thorough review of the mechanism of transcriptional transrepression of PPARs can be found in the literature (Ricote and Glass, 2007).

Ligand-indipendent transrepression

PPARs may repress the transcription of direct target genes in the absence of ligands (ligand-independent repression). PPARs bind to response elements in the absence of ligand and recruit co-repressor complexes that mediate active repression. The co-repressors are capable of fully repressing PPAR-mediated transactivation induced either by ligands or by cAMP-regulated signalling pathways. This suggests co-repressors as general antagonists of the various stimuli inducing PPAR-mediated transactivation. Co-repressors can display different ligand selectivity: the nuclear receptor co-repressor NCoR interacted strongly with the ligand-binding domain of PPAR-β/δ, whereas interactions with the ligand-binding domains of PPAR-γ and PPAR-α were significantly weaker (Krogsdam et al., 2002).

PPARs in the brain

Although PPARs exhibit high homology at the amino acid level and are structurally similar, the tissue distribution varies greatly between the subtypes. PPAR-α is found mainly in the liver, kidney, skeletal, and cardiac muscle, PPAR-β/δ is ubiquitously expressed, whereas PPAR-γ is mainly found in adipocytes and in cells of the immune system such as monocytes/ macrophages, B and T cells, and dendritic cells (Braissant et al., 1996; Chinetti et al., 1998; Chinetti et al., 2000; Clark et al., 2000; Faveeuw et al., 2000; Gosset et al., 2001; Marx et al., 1998; Yang et al., 2000). Interestingly, all three PPAR isotypes are co-expressed in the nervous system during late rat embryogenesis. Their expression peaks in the central nervous system at mid-gestation. Whereas PPAR-β/δ remains highly expressed in this tissue, the expression of PPAR-α and PPAR-γ decreases postnatally in the brain (Braissant et al., 1996). While PPAR-β/δ has been found in neurons of numerous brain areas of adult rodents, PPAR-α and PPAR-γ have been localized to more restricted areas of the brain (Moreno et al., 2004; Woods et al., 2003). As shown by Moreno et al. (2004), the immunohistochemical localization of the three isotypes of PPARs in the CNS of the adult rat demonstrates that some brain areas express all the studied receptors, while others exclusively contain specific isotypes. The former group comprises the basal ganglia, hippocampal formation and many rhombencephalic nuclei; the latter includes the olfactory bulb, lacking PPAR-γ, the hypothalamus, immunonegative to PPAR-α and the spinal cord, generally devoid of PPAR-α. Besides, as reported by Kremarik-Bouillaud et al. (2000), in some regions at the distribution patterns are specific for the cell type. For example, in the cerebellar cortex, Golgi cells display all PPAR isotypes, while Purkinje cells only contain the β isotype. The localization of PPARs has also been investigated in purified cultures of neural cells. PPAR-β/δ is expressed in immature oligodendrocytes where its activation promotes differentiation, myelin maturation and turnover (Cimini et al., 2003; Saluja et al., 2001). The PPAR-γ isotype is the dominant isoform in microglia. Astrocytes possess all three PPAR isotypes, although to different degrees depending on the brain area and animal age (Cristiano et al., 2001; Cullingford et al., 1998). The role of PPARs in the CNS is mainly related to lipid metabolism; however, these receptors have been implicated in neural cell differentiation and death as well as in inflammation and neurodegeneration. The expression of PPAR-γ in the brain has been extensively studied in relation to inflammation and neurodegeneration (Heneka et al., 2000). PPAR-α has been suggested to be involved in acetylcholine metabolism (Farioli-Vecchioli et al., 2001), excitatory amino acid neurotransmission and oxidative stress defense (Moreno et al., 2004). PPAR-β/δ seems to play a critical role in regulating myelinogenesis and differentiation of cells within the CNS (Saluja et al., 2001; Peters et al., 2000). Several lines of evidence from *in vitro* and *in vivo* studies support the hypothesis that PPAR agonists could be potential neuroprotective drugs in neurodegenerative diseases and multiple sclerosis. These data

have been essentially obtained with PPAR-α and PPAR-γ agonists in animal models of Alzheimer's disease, Parkinson disease and experimental allergic encephalitis, an established animal model of multiple sclerosis (Feinstein, 2003).

PPARs and cerebral ischemia/reperfusion injury

Experimental models of cerebral I/R injury

Although the relevance of animal models to the development of therapies for acute stroke has been often questioned, evidence demonstrates that animal models of stroke do have clinical relevance and are useful in the development of drugs that attenuate I/R-induced damage (Green et al., 2003). A role for PPARs in reducing I/R injury has been first established in animal models of acute myocardial infarction (Yue et al., 2001; Wayman et al., 2002). More recently, good evidence supporting the beneficial effects of PPAR agonists in stroke has been provided by several *in vivo* experimental models of cerebral I/R injury. It has been demonstrated that a 14-day preventive treatment with fenofibrate reduced susceptibility to stroke in apolipoprotein E-deficient mice as well as decreased cerebral infarct volume in wild-type littermates (Deplanque et al., 2003). The authors demonstrated that fenofibrate administration was associated with a decrease in cerebral oxidative stress depending on the increase in activity of several anti-oxidant enzymes and with a reduced expression of adhesion molecules. In another study, it was confirmed that two different PPAR-α agonists, fenofibrate and WY14643, provided similar brain protection when administered 3 or 7 days, respectively, before the induction of cerebral ischemia (Inoue et al., 2003). More recently, we have found that PPAR-α agonists may also reduce cerebral I/R injury when administered just before ischemia or during reperfusion (Collino et al., 2006a). Specifically, we reported that administration of the selective PPAR-α agonist, WY14643 decreased reactive oxygen species (ROS) production and lipid peroxidation in the hippocampus of rats subjected to I/R and, at the same time, offered protection from I/R-induced inducible nitric oxide synthase (iNOS) and intercellular adhesion molecule-1 (ICAM-1) overexpression. We showed that the potential neuroprotective effects of PPAR-α agonists is manifested by modulation of protein S100B levels in the rat CNS. S100B is a calcium-binding protein, mainly expressed in the brain and recent clinical studies indicate that increased S100B levels is a reliable indicator of infarct size in patients with stroke (Buyukuysal, 2005). Pre-treatment of rats with the selective PPAR-α agonist, WY14643, prior to cerebral ischemia causes a marked reduction of S100B levels in the rat hippocampus (Figure 1). This protective effect is reversed by administration of the PPAR-α antagonist, MK886,

thus confirming the involvment of PPAR-α activation in neuroprotection. The key role of PPAR-α isotype after focal cerebral ischemia has been further demonstrated by using PPAR-$\alpha^{-/-}$ mice (Pialat et al., 2007). However, the principal focus of studies of PPAR agonists has been on agonists of the PPAR-γ isoform. Emerging studies have reported the protective effects of PPAR-γ agonist administration in animal models of cerebral I/R injury (Allahtavakoli et al., 2007; Allahtavakoli et al., 2006; Shimazu et al., 2005; Sundararajan et al., 2005; Collino et al., 2006b). These neuroprotective effects have been related to the inhibition of I/R-induced inflammatory markers (IL-1 β, iNOS, ICAM-1, cyclooxygenase-2 [COX-2]) and to an anti-oxidant effect (increased expression of superoxide dismutase 1, reduced production of ROS, lipid peroxidation and glutathione [GSH] depletion). In one of these studies, infarct volume was reduced and neurological function was improved by PPAR-γ agonist treatment when measured 22 days after the ischemic event. This suggests that agonist treatment near the time of ischemia, has long term protective effects (Sundararajan et al., 2005). The relevance of PPAR-γ as an endogenous protective factor was also shown by the fact that treatment with a PPAR-γ antagonist increased infarct size (Victor et al., 2006). The intracerebroventricular application of the PPAR-γ agonist pioglitazone has been demonstrated as effective as systemic application, thus indicating that the protection is brought about by the selective stimulation of intracerebral PPAR-γ (Zhao et al., 2005). PPAR-γ mRNA is up-regulated in ischemic brain, especially in the peri-infarct area. Increased PPAR-γ mRNA was detected in the infarcted brain as early as 6 h following focal ischemia (Ou et al., 2006), and PPAR-γ immunopositive neurons were detected between 4 h and 14 days (Victor et al., 2006), whereas in neurons and microglia only transiently at 12 h in the post-ischemic brain (Zhao et al., 2006a and Zhao et al., 2006b). Surprisingly, the increased neuronal PPAR-γ expression was associated with a reduced DNA-binding activity. As reported by Victor and colleagues (2006) ischemia reduced PPAR-γ binding in the ipsilateral hemisphere of the brain, and rosiglitazone treatment increased the binding in general, where it resulted in a more noticeable increase in binding activity on the contralateral side to the injury. Similarly, administration of the natural PPAR-γ ligand, 15d-PGJ_2, resulted in increased binding of PPAR-γ to the PPRE and reduced the area of infarct (Ou et al., 2006).

Recently, the beneficial role of PPAR-β/δ in stroke has been demonstrated by two different studies in which PPAR-$\beta/\delta^{-/-}$ mice subjected to cerebral I/R showed significantly larger infarct size than wild-type littermates (Arsenijevic et al., 2006; Pialat et al., 2007). This finding is confirmed by another study demonstrating that intracerebroventricular administration of high affinity PPAR-β/δ agonists such as L-165041 and GW501516 significantly decreased the infarct volume at 24 h of reperfusion after cerebral ischemia in rats (Iwashita et al., 2007).

Clinical evidence

As already mentioned, pioglitazone and rosiglitazone (TZD class of PPAR-γ agonists) have proven to be beneficial in type-2 diabetes mellitus patients. Diabetics are at an increased risk of stroke incidence and stroke causes more damage in diabetics compared to normoglycemic individuals (Kagansky et al., 2001). The outcome of a large clinical trial (PROactive) has recently been reported and demonstrated that pioglitazone significantly reduces the combined risk of heart attacks, strokes and death by 16% in high risk patients with type-2 diabetes (Dormandy et al., 2005). However, TZDs are hampered by adverse effects related to increased weight gain, fluid overload, and congestive heart failure, so their role in prevention of cardiovascular diseases is not yet fully defined. Recently, enhanced functional recovery was reported in a small group of stroke patients with type-2 diabetes treated with pioglitazone or rosiglitazone (Lee and Reding, 2006). Importantly, high plasma levels of 15d-PGJ_2 (the natural ligand for PPAR-γ) have been associated with good neurological outcome and smaller infarct volume in patients with an acute atherothrombotic stroke (Blanco et al., 2005). Moreover, a recent report suggests that the Pro12Ala polymorphism of PPAR-γ2 is associated with a reduced risk for ischemic stroke (Lee et al., 2006), further supporting the importance of PPARs in cerebral ischemia.

Abnormal levels of serum lipids, including triglycerides, low density lipoprotein (LDL) and high density lipoprotein (HDL), are regarded as other important risk factors for cerebrovascular disease, including stroke. The association between hypercholesterolemia and stroke has become more apparent because of data from prospective cohort studies that show higher risks of ischemic stroke with increasing levels of total cholesterol in both men and women (Iso et al., 1989; Kagan et al., 1980; Leppala et al., 1999; Horenstein et al., 2002). Increased HDL cholesterol levels have a protective effect against the occurrence of ischemic stroke (Soyama et al., 2003; Sacco et al., 2001) and elevated triglyceride levels have also been reported as a risk factor for stroke (Horenstein et al., 2002; Tanne et al., 2001). Overall, elevated total cholesterol confers an approximately two-fold relative increase in stroke risk for men and women (Goldstein et al., 2001). As fibrates are used as lipid-lowering agents, it has been supposed that these PPAR-α agonists could also protect the brain against noxious biological reactions induced by cerebral ischemia/reperfusion (I/R). A very recent systematic meta-analysis of randomized clinical trials shows that fibrates do not significantly reduce the odds of stroke (Saha et al., 2007). However, data from large trials specifically investigating the role of fibrates in stroke event reduction are needed to conclusively elucidate their potential neuroprotective role. For instance, a large clinical trial, named Action to Control

Cardiovascular Risk in Diabetes (ACCORD) is currently testing the ability of fenofibrate to decrease stroke incidence in high-risk patients with type-2 diabetes (ACCORD study group 2007).

Mechanisms of beneficial effects of PPARs against cerebral ischemia/reperfusion injury

Cerebral I/R is known to induce generation of ROS, as well as the expression of cytokines, adhesion molecules and enzymes involved in the inflammatory response, and is known to be regulated by oxygen- or redox-sensitive mechanisms (Dirnagl et al., 1999). Recent studies have confirmed the pivotal role of both oxidative stress and inflammatory response in the pathogenesis of acute ischemic stroke (del Zoppo, 2006; Schaller 2005). Through various mechanisms PPARs can regulate both inflammatory and oxidative pathways and PPAR agonist-induced neuroprotection seems to be specific for injuries in which inflammation or free radical generation are the main causes of cell damage. For instance, PPAR-α activation can induce expression and activation of antioxidant enzymes, such as superoxide dismutase (SOD) and glutathione peroxidase (GSH). We have demonstrated that administration of a highly selective PPAR-α agonist, WY14643, 30 min prior to I/R, decreased ROS production and lipid peroxidation in rats subjected to I/R and, at the same time, offered protection against GSH depletion (Collino et al., 2006a). Similar results on oxidative stress modulation have been reported when another PPAR-α agonist, fenofibrate, was tested in a mouse model of middle cerebral artery occlusion (Deplanque et al., 2003). Interestingly, PPAR-$\alpha^{-/-}$ mice have been found to exhibit significant increases in oxidative stress and lipid peroxidation much earlier in their life than wild-type littermates (Poynter, et al., 1998). The PPAR-induced protective effect on oxidative stress could be related to a direct effect on antioxidant enzyme expression, as the catalase and SOD gene promoters contain the PPRE (Moraes et al., 2006, Girnum et al., 2002; Hwang et al., 2005). In fact, rats that have been treated with a diet containing PPAR-α ligands, WY14,643 or fenofibrate, have demonstrated an enhanced expression of antioxidant enzymes such as SOD and catalase (Toyama et al., 2004). Based on gene expression microarray experiments, Coleman and colleagues (2007) demonstrated that PPAR-β/δ activation increased mRNA for aldheyde dehydrogenase and glutathione-S-transferase, thus protecting the cell from oxidative damage. In normotensive and hypertensive animals treated with rosiglitazone, ischemic hemispheres showed increased catalase and Cu/Zn-SOD activity in the peri-infarct region (Tureyen et al., 2007) and the level of Cu/Zn-SOD was demonstrated to increase in the ischemic cortex of animals treated with pioglitazone for 4 days prior to focal cerebral ischemia (Shimazu et al., 2005). As we have recently shown, treatment of rats with either pioglitazone or rosiglitazone

before occlusion of the common carotid artery decreased the production of ROS and nitrite, decreased lipid peroxidation and reversed the depleted stores of glutathione in the hippocampus (Collino et al., 2006b). These findings are supported by data from an *in vitro* model demonstrating that pre-treatment with PPAR-γ agonists protected an immortalized mouse hippocampal cell line against oxidative stress induced by glutamate or hydrogen peroxide (Aoun et al. 2003). Moreover, PPAR-γ agonists attenuate the expression of iNOS in inflammatory cells (Sundararajan et al., 2995; Pereira et al., 2005), which is an important source of nitric oxide (NO). NO may react with ROS to produce peroxynitrites, with deleterious effects on neuronal survival. Thus, iNOS inhibition may represent a further mechanism for neuroprotection by PPAR agonists. Mitochondria are the major source of ROS, which are mainly generated at complexes I and III of the respiratory chain (Kudin et al., 2005). There is now evidence indicating that rosiglitazone and pioglitazone exert direct and rapid effects on mitochondrial respiration, inhibiting complex I (Brunmair et al., 2004) and complex III (Dello Russo et al., 2003) activity. As PPAR-γ agonists partially disrupt the mitochondrial respiratory chain, both electron transport and superoxide anion generation are affected. Moreover, a novel mitochondrial target protein for PPAR-γ agonists ("mitoNEET") has recently been identified (Colca et al., 2004). MitoNEET was found associated with components of complex III, suggesting how binding of PPAR-γ agonists to mitoNEET could selectively block different mitochondrial targets. The ability of PPAR-γ agonists to influence mitochondrial function might contribute to their inhibitory effects on ROS generation evoked by I/R.

Another mechanism through which PPAR agonists may provide neuroprotection is by down-regulating inflammatory response associated with I/R. Depending on the affected tissue and which PPAR isoforms are involved, PPAR agonists can differently modulate the intensity, duration and consequences of inflammatory events. For instance, ischemia-induced COX-2 overexpression is prevented by PPAR-γ agonists but not by PPAR-α agonists (Zhao et al., 2006b; Collino et al., 2006a; Sundararajan et al., 2005; Collino et al., 2006b). Activation of PPAR-γ attenuates the expression of matrix metalloproteinase (MMP)-9 and various inflammatory cytokines in ischemic brain tissue (Luo et al., 2006; Pereira et al., 2005). PPAR-γ is constitutively expressed in macrophages and microglial cells (Bernardo et al., 2000) and the systemic treatment of rodents with rosiglitazone reduces the infiltration of these cells into peri-infarct brain regions (Luo et al., 2006; Sundararajan et al., 2005). Both chronic and acute administration of PPAR-α agonists has been demonstrated to prevent cerebral I/R-induced expression of vascular cell adhesion molecule-1 (VCAM-1) and ICAM-1 in two independent studies (Deplanque et al. 2003; Collino et al., 2006a). In the brain, the decreased expression of these adhesion molecules might contribute to inhibit the infiltration of the brain ischemic area by neutrophils (Lee et al., 2000; Chan, 2001).

Studies addressing the molecular mechanisms of these anti-inflammatory actions demonstrated that the involvement of PPARs in the control of I/R-induced inflammation is mediated mainly through their transrepression capabilities. PPARs can suppress the activities of many distinct families of transcription factors. The range of transcription factors affected and the mechanisms involved may be different for each PPAR isotype, although a common mechanism of PPAR-α and PPAR-γ neuroprotection appears to involve, inhibition of p38 MAPK activation and NF-κB, nuclear translocation (Figure 2). A recent study confirmes that PPAR-γ activation prevents the post-ischemic cerebral expression of pro-inflammatory transcription factors, such as Egr1, C/EBPβ and NF-κB, possibly by decreasing DNA binding (Tureyen et al., 2007). The inhibitory protein IκBα, which is an indicator of NF-κB transcriptional activity, is remarkably increased in the brain of rats that underwent cerebral ischemia and completely blocked by rosiglitazone and 15d-PGJ_2 administration, thus further confirming that both endogenous and synthetic PPAR-γ ligands inhibit NF-κB signalling (Pereira et al., 2006). Similarly, p38 MAPK and NF-κB activation by cerebral I/R has been demonstrated to be inhibited by pre-treatment with the PPAR-α agonist WY14643 or the PPAR-γ agonist pioglitazone (Figure 2). However, as MAPK and NF-κB are functionally interconnected and do not act independently (Carter et al., 1999; Vanden Berghe et al., 1998), we cannot rule out the possibility that PPARs affect NF-κB activation by interfering with the MAPK signalling cascade or vice versa (Figure 3).

The generation of ROS is known to be associated with the induction of apoptosis and, in neurons, inhibition of cell death is an important factor to prevent I/R injury. PPAR activation may decrease the I/R-induced activation of apoptotic pathways depending on the increase in activity and expression of numerous anti-oxidant enzymes. Moreover, by their anti-inflammatory action on miocroglia and astrocytes, PPAR agonists prevent the release of neurotoxic agents, which induce neuronal apoptosis (Combs et al., 2000). For instance, pioglitazone prevented ischemia-induced increase in pro-apoptotic Bax, while increasing anti-apoptotic Bcl-2 expression in the peri-infarct area following focal ischemia (Sakamoto et al., 2000; Sulejczak et al., 2004). Chu and colleagues (2006) showed that rosiglitazone-fed rats had better neurological scores and reduced number of TUNEL-positive cells following transient focal ischemia. Interestingly, these authors also reported an increased vasculature in the rosiglitazone-treated group with increased number of endothelial cells positive for BrdU, suggesting there may be enhanced angiogenesis following PPAR-γ activation. Administration of a selective PPAR-γ agonist (L-796449) 10 min prior to permanent cerebral artery occlusion, resulted in decreased apoptosis, measured as reduction of caspase-3 activity (Pereira et al., 2005). Another study confirmed inhibition on caspase-3 activity by both exogenous and endogenous PPAR-γ agonists, rosiglitazone and 15d-PGJ_2, in the ischemic cortex

(Lin et al., 2006). The same authors observed that rosiglitazone and 15d-PGJ_2 exhibit a concentration-dependent paradoxical effect on cytotoxicity, when tested in an *in vitro* model of hydrogen peroxide induced neuronal apoptosis. The drugs induced pro-apoptotic effects when used at concentrations higher that 5 μmol/L but protect neurons from necrosis and apoptosis at concentrations lower than 1 μmol/L. The reason for this paradoxical action is unclear and further studies are needed to better clarify the effects of PPARs in I/R induced-apoptosis and necrosis.
Recently published data suggest that an increased uptake of cerebral extracellular glutamate levels after ischemia may represent an additional mechanism for the neuroprotection exerted by PPAR-γ activation (Romera et al., 2007). Both *in vivo* and *in vitro* experiments showed that rosiglitazone administration increased the expression of the GLT1/EAAT2 glutamate transporter in the brain, thus preventing the extracellular glutamate levels from rising to neurotoxic values.

Concluding remarks

Although clinical data are limited, a wide array of evidence obtained in animal models now shows that PPAR activation may be a rational and effective strategy against ischemic brain damage. The beneficial effects of PPAR agonists in experimental models of stroke are mediated by different mechanisms, as expected based on their pleiotropic pharmacological profile. The neuroprotective actions appear to be mainly related to the reduction in oxidative damage as well as anti-inflammatory and anti-apoptotic effects (Figure 3). These results have been essentially obtained with PPAR-α and PPAR-γ agonists, while the PPAR-δ/β pathway remains largely unexplored, despite a significant interest in this target. Selective activation of different isoforms of PPARs may account for the difference in molecular pathways underlying neuroprotection and these different features still remain far from being completely understood. In conclusion, currently available management protocols for patients with stroke may benefit from the use of PPAR agonists that target detrimental processes associated with I/R injury. However, critical issues still wait to be resolved. For instance, well-structured clinical trials aimed to evaluate the effects of PPAR ligands on stroke recovery are needed before firm conclusions are drawn about their therapeutic efficacy. A more stringent approach regarding the concentration range of PPAR agonists, especially within the CNS, and the duration of exposure should be applied. Also acceptable water solubility with satisfactory blood-brain barrier penetrability is an important aspect of PPAR agonists that needs to be optimized.

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Figures Legend

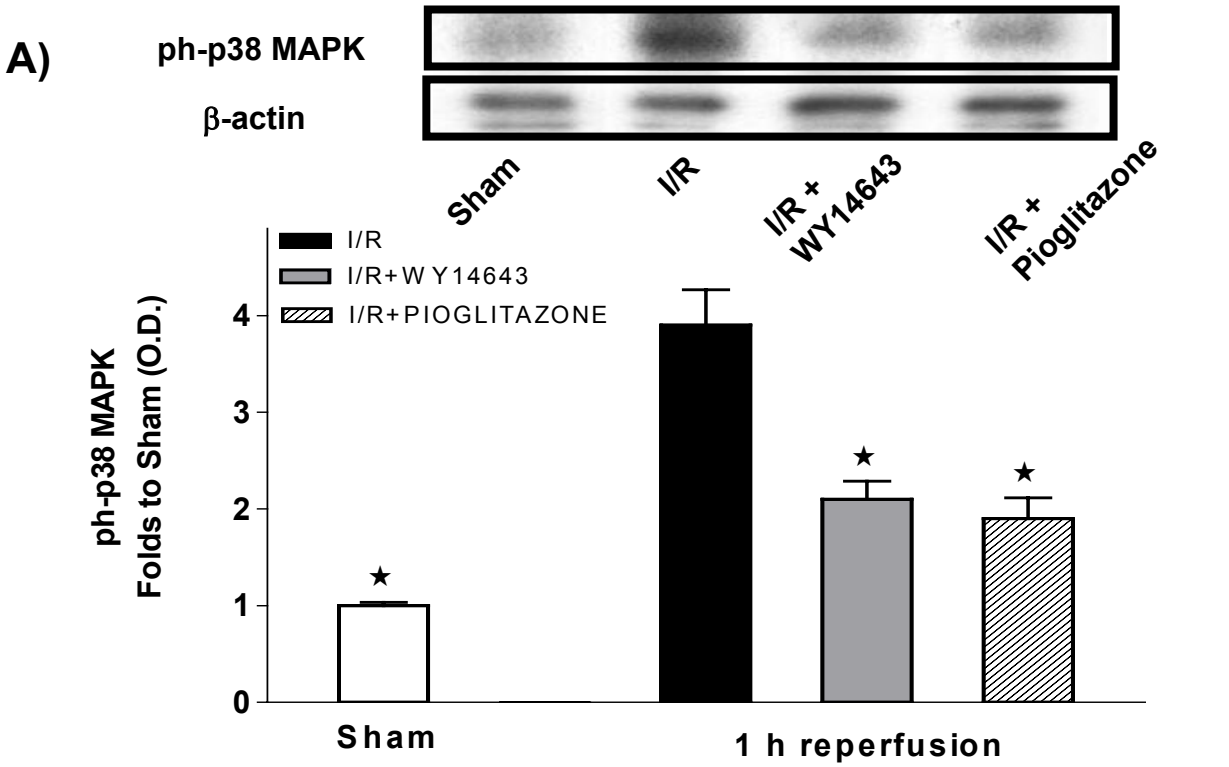
Figure 1 The PPAR-α agonist WY14643 protects against the I/R-induced overexpression of S100B, a calcium binding protein, which has been recognized as marker of neuronal damage. Rats were administered 6 mg/kg WY 14643 (I/R + WY14643) 30 min prior to cerebral I/R. A group of rats was pretreated with both the selective PPAR-α antagonist MK886 (6 mg/kg) and the PPAR-α agonist WY14643 WY14643 (6 mg/kg) before I/R (I/R + WY14643 + MK886). Protein levels were detected in the rat hippocampus homogenates after 30 min ischemia followed by 6 h reperfusion (Panel A). Densitometric analysis of the related bands is expressed as relative optical density (O.D.) of the bands, corrected for the corresponding β-actin contents and normalised using the related sham-operated band (Panel B). Densitometry results are expressed as means ± S.E.M. of three separate experiments. Statistical analysis: ★ $p < 0.01$ versus I/R.. (*Modified from Collino et al., 2006a*)

Figure 2 Effect of pre-treatment with the PPAR-α agonist, WY14643, and the PPAR-γ agonist, pioglitazone, on phosphorylation of p38 MAPK (Panel A) and nuclear translocation of p65 NF-κB (Panel B) evoked by cerebral I/R injury. Phosphorylated p38 MAPK was detected at 1, h reperfusion in rat hippocampus homogenates. NF-κB translocation from the cytosol to the nucleus was evaluated at the same reperfusion time, measuring NF-κB p65 subunit levels in both cytosol and nuclear fractions and expressing the results as nucleus/cytosol ratio (Panel B). Rats were administered 6 mg/kg WY14643 (I/R+WY14643) or 1 mg/kg pioglitazone (I/R+Pioglitazone) before 30 min ischemia. Densitometric analysis of the related bands is expressed as relative optical density (O.D.) of the bands, corrected for the corresponding β-actin contents and normalised using the related sham-operated band. Densitometry results are expressed as means ± S.E.M. of three separate experiments. Statistical analysis: ★ $p < 0.01$ versus I/R. (*Modified from Collino et al., 2006a and Collino et al., 2006b*)

Figure 3 Multiple targets for PPARs in cerebral I/R-induced injury. The tissue injury associated with cerebral I/R results in the activation of MAPKs and transcription factors (including NF-kB, AP-1, STAT, NFAT). The stimulation of PPARs by selective agonists in brain tissues evokes multiple effects that result in regulation of the MAPK cascade and inhibition of transcription factors activation (Figure 3a). As shown in Figure 3b, PPARs activation may cause a functional inhibition

of proteins of the NF-kB family, such as p50 and p65, thus preventing them from inducing the transcription of genes involved in the oxidative stress pathway and in the inflammatory and apoptotic response, all of which may further aggravate the tissue injury (initial insult).

Figure 1


A)
ph-p38 MAPK
β-actin
Sham
I/R
I/R + WY14643
I/R + Pioglitazone
I/R
I/R+WY14643
I/R+PIOGLITAZONE
ph-p38 MAPK
Folds to Sham (O.D.)
0
1
2
3
4
Sham
1 h reperfusion

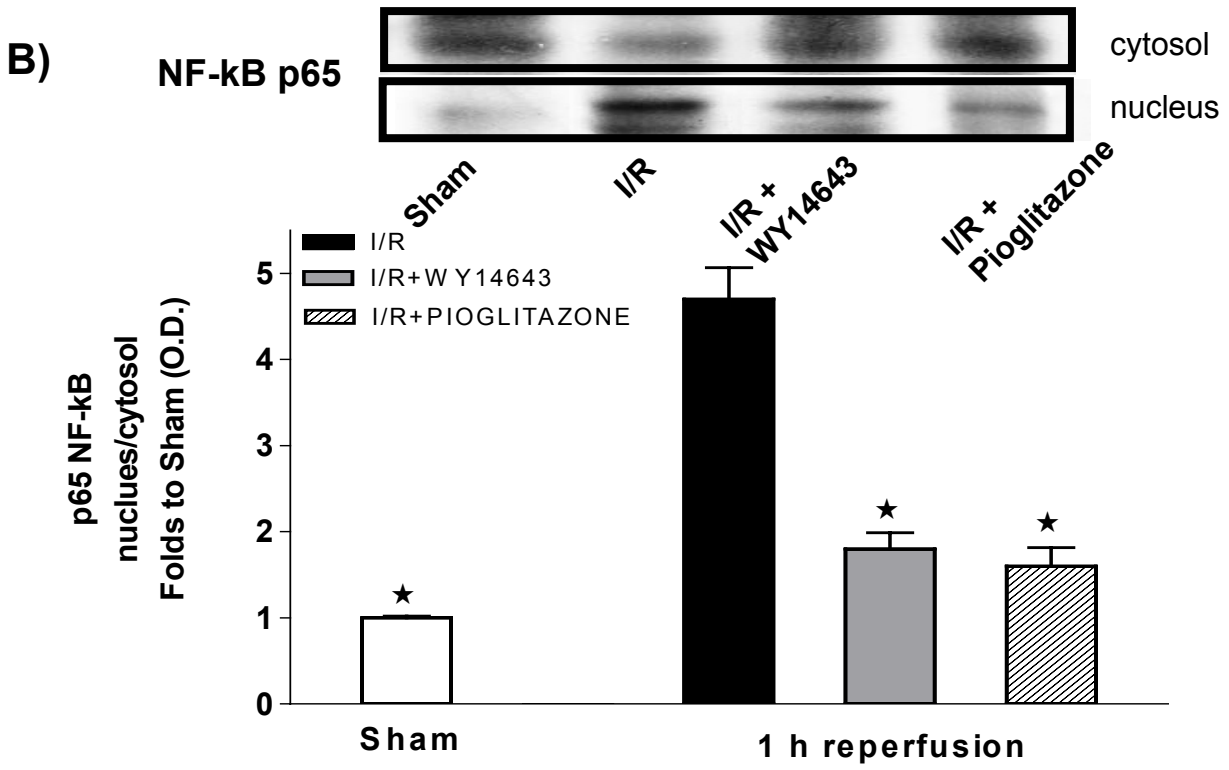
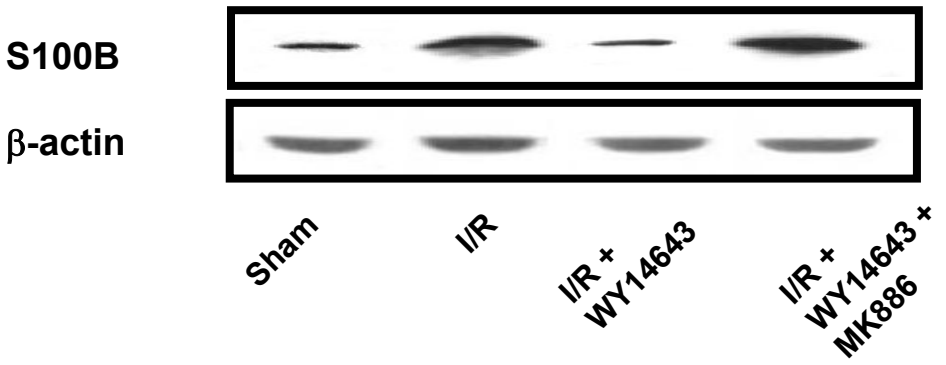

B)
NF-kB p65
cytosol
nucleus
Sham
I/R
I/R + WY14643
I/R + Pioglitazone
I/R
I/R+WY14643
I/R+PIOGLITAZONE
p65 NF-kB
nuclues/cytosol
Folds to Sham (O.D.)
0
1
2
3
4
5
Sham
1 h reperfusion

Figure 2

A)


S100B
β-actin
Sham
I/R
I/R + WY14643
I/R + WY14643 + MK886

B)

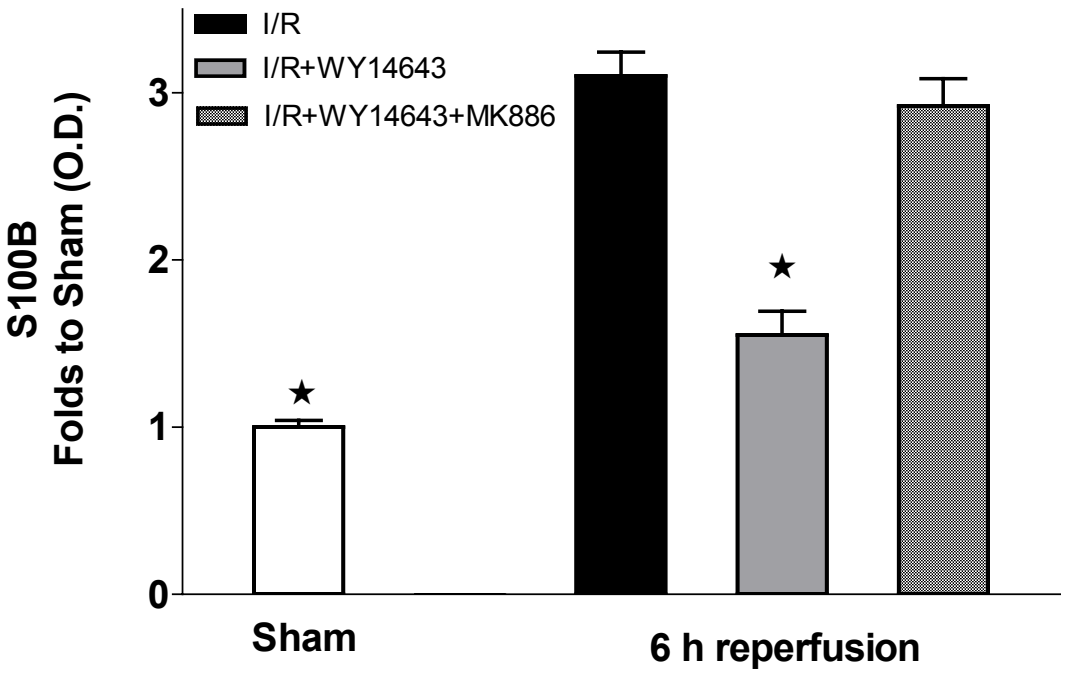
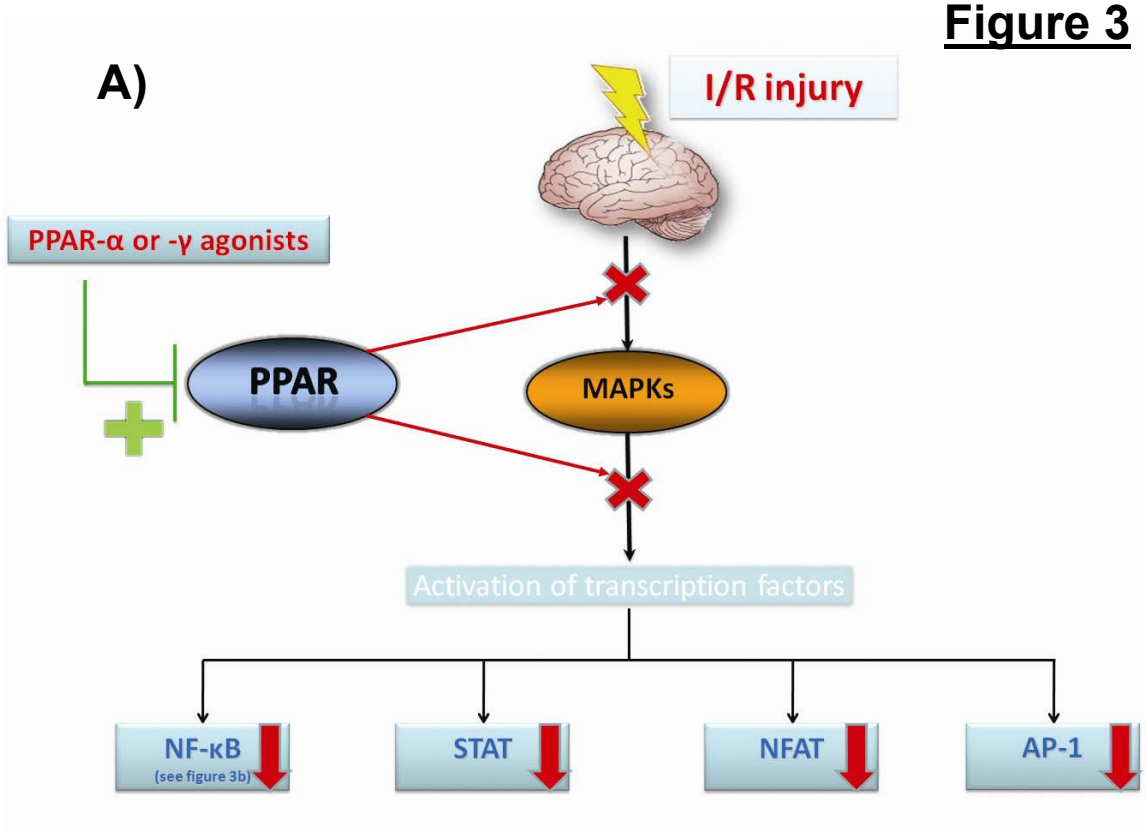
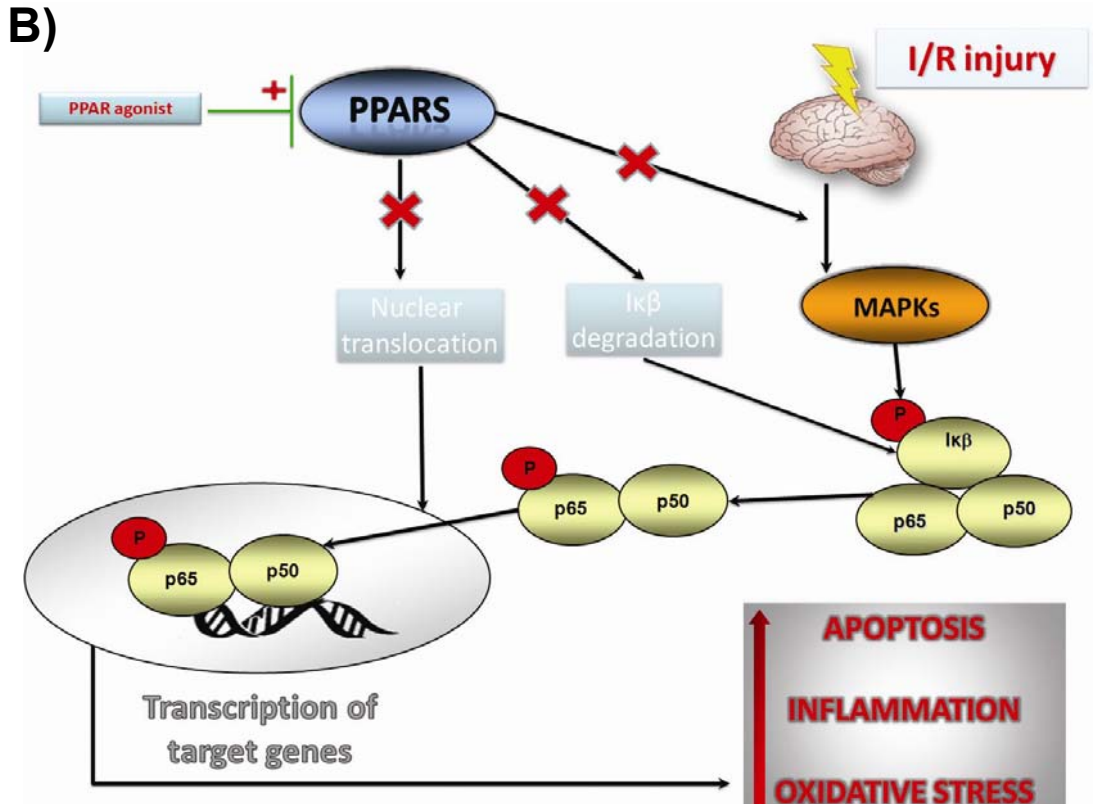

I/R
I/R+WY14643
I/R+WY14643+MK886
S100B
Folds to Sham (O.D.)
0
1
2
3
★
★
Sham
6 h reperfusion

Figure 3


A)
I/R injury
PPAR-α or -γ agonists
PPAR
MAPKs
Activation of transcription factors
NF-κB
(see figure 3b)
STAT
NFAT
AP-1


B)
PPAR agonist
PPARS
I/R injury
Nuclear translocation
Iκβ degradation
MAPKs
P
Iκβ
p65
p50
Transcription of target genes
APOPTOSIS
INFLAMMATION
OXIDATIVE STRESS